Anionic phospholipids can mediate membrane insertion of the anionic part of a bound peptide

J.M. Leenhouts*, P.W.J. van den Wijngaard, A.I.P.M. de Kroon, B. de Kruijff

Department of Biochemistry of Membranes (CBLE), Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

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Abstract The effect of anionic lipids on the membrane insertion of a carboxyl group on a specially designed palmitoylated peptide was studied, using tryptophan fluorescence. It is demonstrated that the negatively charged membrane surface of mixed phosphatidylcholine/phosphatidylglycerol small unilamellar vesicles enhances the protonation of the C-terminal carboxyl group, and the subsequent insertion of that part of the peptide.

Key words: Anionic phospholipid; Peptide insertion; Fluorescence spectroscopy; Surface pH

1. Introduction

Anionic phospholipids are involved in various ways in the membrane interaction of proteins (for a recent review see [1]). The roles of anionic lipids in membrane binding and insertion of proteins include in the first place electrostatic interactions between the negatively charged lipid headgroups and positively charged protein residues. Such interactions can lead for example to the proper assembly of protein complexes on the membrane surface. Alternatively, such electrostatic interactions can enable subsequent insertion of the polypeptide into the membrane. A third, more hypothetical function of anionic lipids in membrane insertion of proteins is related to the locally lower interfacial pH, due to accumulation of protons at the negatively charged membrane surface. This could in principle stimulate protonation of carboxylate-rich domains, which then become more hydrophobic. Since electrostatic repulsion will prevent approximation of anionic protein domains to the negatively charged membrane surface, membrane anchoring of carboxylate-rich protein domains, via other parts of the protein, is required for the anionic lipids to exert this possible function.

This hypothetical role of anionic phospholipids has particularly been suggested in studies on membrane insertion of protein toxins. For example, the pore-forming domains of colicins require a low pH and negatively charged lipids for membrane insertion [2,3]. Other examples are the membrane penetration processes of diphteria toxin and tetanus toxin, which both depend on pH and lipid composition of the membrane [4,5].

The present study aims at directly testing the hypothesis that anionic lipids enhance the membrane penetration of a negatively charged group on a peptide by protonation of that group. For this purpose, two peptides were designed: H_3N^+-Arg-Ala-Gly-Ala-Trp-Ala-$CONH_2$ and H_3N^+-Arg-Ala-Gly-Ala-Trp-Ala-COO^- referred to as p-$CONH_2$ and p-COO^-, respectively. The open carboxyl group is used as a model for negatively charged residues, like aspartic acid and glutamic acid, which also have carboxyl groups, with pK values of 3.9 and 4.1, respectively [6]. The pK of the terminal carboxyl group of a peptide is influenced by the peptide linkage and usually varies between 3.1–3.8 [7]. The advantage of this group as a model is that the identical peptide with a closed C-terminus can be used as a reference peptide, representing a permanently protonated state. In order to anchor the positively charged arginine residue of these peptides at the membrane–water interface, a palmitic acid was covalently coupled to the N-terminus of these peptides, resulting in C_{16}p-$CONH_2$ and C_{16}p-COO^-, respectively. The effect of protonation of C_{16}p-COO^- on its membrane insertion is monitored by fluorescence spectroscopy, using the tryptophan residue as an intrinsic reporter group, which is located only one residue away from the carboxyl group.

2. Experimental

2.1. Peptides

The two hexapeptides H_3N^+-Arg-Ala-Gly-Ala-Trp-Ala-$CONH_2$ and H_3N^+-Arg-Ala-Gly-Ala-Trp-Ala-COO^-, were synthesized on an Excell Pepsynthesizer by the Hubrecht Laboratories (Utrecht, The Netherlands). The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a 250 × 10 mm RP8 column (Merck, Darmstadt, Germany) using linear water-acetonitrile gradients, containing 0.1% (v/v) trifluoroacetic acid as eluent. The peptides eluted both at 27% acetonitrile.

Covalent coupling of a palmitic acid to the amino terminus of either peptide was performed essentially according to Lapidot et al. [8]. In short, equimolar amounts of the N-hydroxysuccinimide ester of palmitic acid (Sigma Chemical Co., St. Louis, MO, USA) and the peptide were mixed in tetrahydrofuran (THF)/water. An equimolar amount of $NaHCO_3$ and a fourfold molar excess of triethylamine were added, resulting in a final mixture of THF/water (2:1, v/v). The mixture was allowed to react for at least 42 h at room temperature under magnetic stirring. Subsequently, the palmitoylated peptide was isolated by HPLC as described above. The yield of this coupling was 65% for C_{16}p-COO^-, and 41% for C_{16}p-$CONH_2$, based on weight. The identity of all peptides was confirmed by mass spectroscopy.

Stock solutions of the peptides were made in water/ethanol (1:1, v/v). The concentrations of the stock solutions were determined by absorption at 280 nm, using ε^{280} (in water) = 5600 $M^{-1} \cdot cm^{-1}$.

2.2. Vesicle preparation

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Phospholipid stock solutions were made in chloroform. Dry mixed lipid films were made by mixing the appropriate amounts of DOPC and DOPG stock solutions and subsequently evaporating the chloroform under reduced pressure. Dry mixed films of lipid and either one of the acylated peptides were made by mixing the desired amounts of phospholipid and acylated peptide stock solutions (lipid/peptide 500:1 molar ratio), and adding iso-

*Corresponding author. Fax: (31) (30) 522 478.

Abbreviations: aa, acrylamide; SUVs, small unilamellar vesicles; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; HPLC, high-performance liquid chromatography.

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propanol until a clear and homogenous solution was obtained. The solvents were evaporated under reduced pressure at room temperature, and the resulting film, which was visually homogenous, was dried for at least 2 h under high vacuum over P_2O_5.

Small unilamellar vesicles (SUVs) were made by hydrating a dry lipid or mixed lipid/peptide film (corresponding to 13 μmol lipid) in 0.5 ml buffer, containing 100 mM NaCl, 10 mM each of HEPES, MES and glutamic acid (abbreviated as 10 mM HMG), pH 8.0, followed by 5 times freezing and thawing, and subsequently 10 times 30 s of sonication. Sonication was carried out under nitrogen, while cooling on ice, at 50 W using a Branson Sonifier 250 equipped with a tip sonicator. After sonication, the solution of SUVs was centrifuged for 20 min at $37{,}000 \times g$ in order to remove titanium particles.

Phospholipid concentrations were determined according to Rouser et al. [9].

2.3. Fluorescence measurements

Tryptophan fluorescence emission spectra were measured from 300–400 nm on an SLM-Aminco SPF-500C fluorometer, at 22°C under magnetic stirring, using an excitation wavelength of 280 nm (band passes 5 nm). All spectra were corrected for scattering, using a vesicle blank, and for dilution.

The effect of the bulk phase pH on the tryptophan emission spectrum was determined by varying the pH of a 1.2 ml buffer solution (100 mM NaCl, 10 mM HMG, pH 8.0), containing SUVs (1.6 mM of lipid) with either one of the acylated peptides incorporated. Each point of the titration was obtained by recording a spectrum 5 min after adjusting the pH of the buffer to the desired value with 1 M HCl. The reversibility of the observed changes was determined by increasing the pH with 1 M NaOH.

Quenching of tryptophan fluorescence by acrylamide (aa) was determined by measuring the tryptophan fluorescence emission spectrum of a SUVs solution (1 mM of lipid), after the addition of increasing amounts of a 3 M acrylamide solution. Spectra were recorded on a Perkin Elmer L550B luminescence spectrometer, under continuous stirring at 22°C, using an excitation wavelength of 295 nm instead of 280 nm, in order to reduce the absorbance by acrylamide. The spectra were corrected for scattering, using a vesicle blank without the acylated peptide in the presence of acrylamide, for dilution, and for the inner filter effect of acrylamide. The data were analyzed according to the Stern–Volmer equation for collisional quenching [10]: $F_o/F = 1 + K_{sv}$.[aa], where K_{sv} is the quenching constant and F_o and F are the fluorescence intensities in the absence and presence of quencher, respectively.

3. Results and discussion

Changes in the tryptophan fluorescence emission spectrum of the peptides were monitored in order to get insight into the localization of the tryptophan residue, and thus the C-terminus, of the peptide. Both a blue shift of the λ_{max} and an increase in the fluorescence quantum yield indicate the entry of the tryptophan in a more hydrophobic environment, which is interpreted as a deeper insertion into the bilayer. To ensure that the spectral changes are due to changes in localization and not to increased binding of the peptide, the palmitoylated peptides were pre-incorporated in the SUVs. The incorporated peptide is expected to be located primarily in the strongly convex curved outer leaflet of the SUVs, because of the wedge shape of the acyl peptide with its bulky peptide headgroup. To get a first indication of the fluorescence response of C_{16}p-COO^- to a decrease of the pH, the effect of the bulk pH on the insertion of the tryptophan residue of C_{16}p-COO^-, incorporated in PC membranes, was investigated. Since SUVs are rather permeable to protons under the experimental conditions used [11], adjusting the pH of the buffer is considered to occur both on the outside and on the inside of the SUVs.

Upon decreasing the pH of the buffer from pH 8.0 to pH 2.8 the λ_{max} of the tryptophan emission spectrum of C_{16}p-COO^-, incorporated in DOPC SUVs is blue-shifted from 351 to 348 nm (not shown). As a result, the fluorescence intensity at 330 nm ($F330$) increases with decreasing pH. This is depicted in Fig. 1 where the fluorescence intensity, relative to that at pH 8.0 ($F/F_0(330)$), is plotted against the pH of the medium. The λ_{max} of the emission spectra of C_{16}p-$CONH_2$, incorporated in DOPC SUVs, did not change as a function of pH and was found to be 348 nm (data not shown). Accordingly, the fluorescence intensity of C_{16}p-$CONH_2$ appeared to be independent on the bulk pH. In control experiments, the pH of buffer solutions, containing the non-acylated peptides, was decreased, resulting in a small decrease in $F/F_0(330)$ for p-$CONH_2$. Apparently, the pH decrease has a small quenching effect on the tryptophan fluorescence intensity that is not related to membrane insertion. The p-COO^- peptide free in buffer showed a much larger decrease in fluorescence quantum yield upon lowering the pH. This is most likely a direct effect of protonation of the carboxyl group on the quantum yield of the neighbouring tryptophan residue (cf. [12]). All changes in the fluorescence spectra were reversed upon increasing the pH back to pH 8.0.

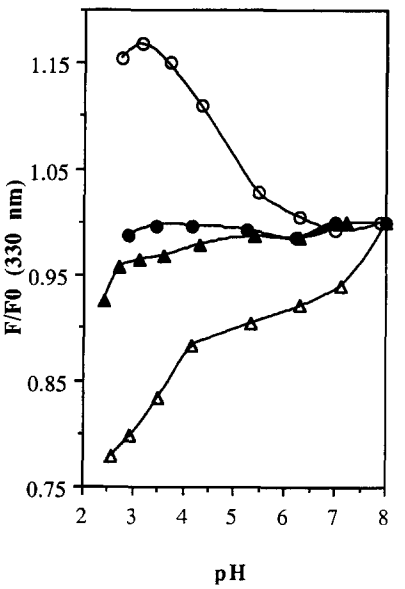

Fig. 1. Tryptophan fluorescence enhancement at 330 nm as a function of pH, relative to the fluorescence at pH 8.0 (F_0). After each stepwise addition of 5 μl 1 M HCl, the pH was measured and, after 5 min incubation, the fluorescence intensity was read at 330 nm (F). Data are shown for PC/C_{16}p-COO^- (○) and PC/C_{16}p-$CONH_2$ (●) SUVs, in 100 mM NaCl, 10 mM HMG buffer, at the indicated pH, and for buffer solutions, containing 7 μM non-acylated p-COO^- (△) and p-$CONH_2$ (▲).

Upon correcting the changes in $F/F_0(330)$ of the SUV-incorporated acylated peptides for the not membrane insertion-related changes of tryptophan fluorescence intensity observed for the corresponding free peptides, an even more pronounced difference is observed between C_{16}p-COO^- and C_{16}p-$CONH_2$ (Fig. 2). The large increase of $F/F_0(330)$ at low pH values for C_{16}p-COO^- reflects a movement of the tryptophan residue, and thus of the carboxyl terminus of the peptide, towards the membrane interior of the PC SUVs. This observation, together with the fact that no such changes were observed for C_{16}p-$CONH_2$, indicate that the deeper insertion is a result of protonation of the open carboxyl terminus of C_{16}p-COO^-. Upon protonation

of the carboxyl group, C_{16}p-COO^- is able to penetrate deeper into the membrane with its C-terminus, resulting in a localization which is similar to that of C_{16}p-$CONH_2$, since λ_{max} shifts from 351 at pH 8.0 to 348 nm at pH 2.8, which is the same as the λ_{max} value found for C_{16}p-$CONH_2$ in the entire pH range (not shown). From the titration curve depicted in Fig. 2, an apparent pK of approx. 4.2 can be derived for the protonation of the open carboxyl terminus of C_{16}p-COO^-, which is close to the predicted value.

In order to investigate whether a negatively charged membrane surface will enhance the insertion of the carboxyl group into the bilayer, PC/PG (85/15 or 50/50 molar ratio) SUVs were prepared, containing either one of the palmitoylated peptides. The negatively charged PG, with a pK of around 3.0 [13], is expected to increase the local interfacial pH, thereby causing protonation, and thus insertion, of the peptide carboxylate group to occur at higher bulk phase pH. Again the fluorescence intensity was read as the pH of the bulk phase was decreased from 8.0 to 2.6. The pH titration curves are shown in Fig. 2 and were corrected for the direct pH effects on the tryptophan fluorescence of the corresponding peptides free in solution. For the PC/PG SUVs, these corrections were made with the assumption that the tryptophan is present at approximately 7.5 Å from the membrane surface, where the calculated local pH is 0.4 and 0.7 pH-units smaller than the bulk phase pH, for SUVs with 15 or 50 mol% PG incorporated, respectively (according to the Gouy–Chapman theory, as described in [14]). For C_{16}p-COO^-, in all cases an increasing relative fluorescence quantum yield was observed upon decreasing the pH, indicating a deeper insertion into the membrane. Comparing the titration curves of C_{16}p-COO^- incorporated in either PC or PC/PG SUVs, a shift in the apparent pK is observed for PC/PG SUVs to a higher bulk pH value. This shift is approximately 0.7 units

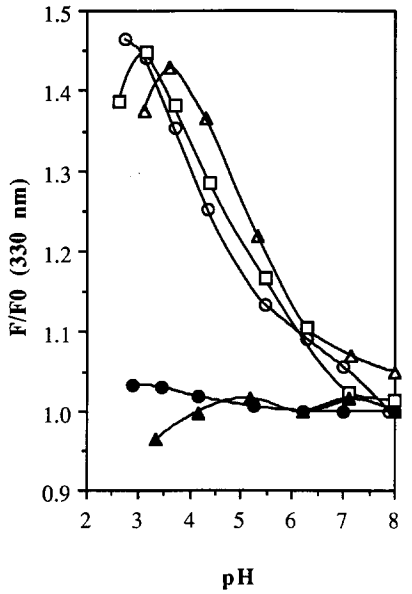

Fig. 2. pH-dependent enhancement in tryptophan fluorescence at 330 nm relative to the fluorescence intensity at pH 8.0 (F_0). Measurements were performed as described in the legend of Fig. 1. Data are depicted for SUVs consisting of PC/PG/C_{16}p-COO^- (open symbols), and PC/PG/C_{16}p-$CONH_2$ (closed symbols) SUVs. Circles: 0 mol% PG; squares: 15 mol% PG and triangles: 50 mol% PG. All values are corrected for pH-dependent changes in tryptophan fluorescence, unrelated to membrane insertion (see text for details).

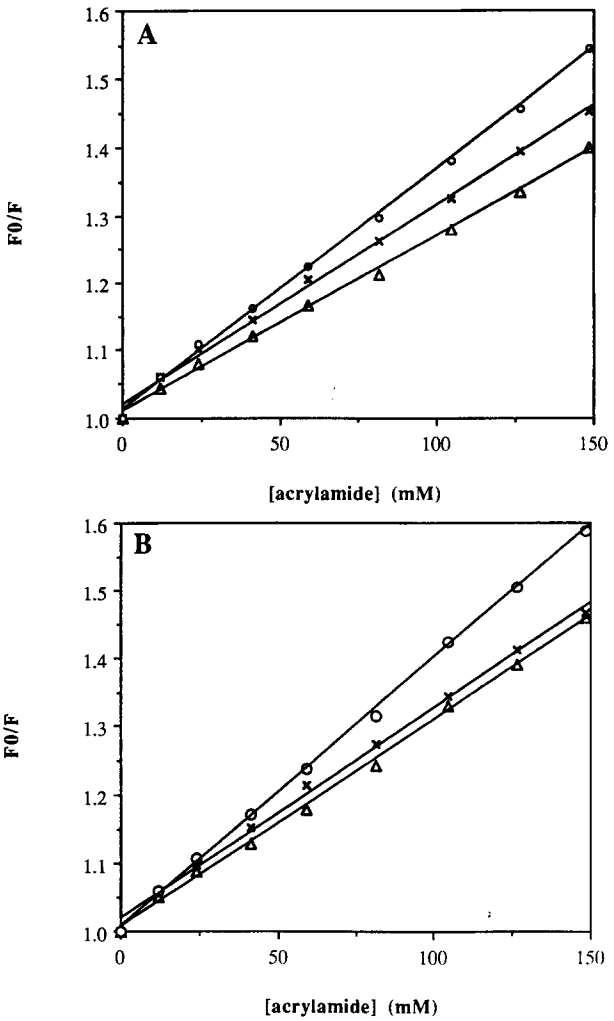

Fig. 3. Stern–Volmer plots of aqueous quenching by acrylamide of C_{16}p-COO^- incorporated in PC SUVs (A) or PC/PG (1:1 molar ratio) SUVs (B). Quenching experiments were performed at a lipid concentration of 1 mM, at pH 8.0 (○), pH 4.7 (×) or pH 3.6 (△).

for the SUVs containing 50 mol% PG, while for the SUVs with the lower PG-content a smaller shift in apparent pK is observed (0.3 units). These values are the same when no corrections for the local pH are made. For C_{16}p-$CONH_2$ no changes in the fluorescence spectra were observed. Apparently, the insertion of the negatively charged group of the C_{16}p-COO^- peptide is enhanced by a decreased local pH at the surface of the PC/PG SUVs, in relation to the amount of negatively charged lipids.

To verify that the observed changes in the tryptophan fluorescence are indeed the result of the tryptophan inserting into the membrane, the accessibility of the tryptophan from the aqueous phase was determined under a number of different circumstances. For this purpose acrylamide was used as the aqueous quencher of tryptophan fluorescence. First, the accessibility of C_{16}p-COO^- incorporated in PC SUVs was studied at different bulk phase pH values, as shown in the Stern–Volmer plots depicted in Fig. 3A. After decreasing the pH to a value of 4.7, which is slightly above the pK of the protonation of the carboxyl group (see Fig. 2), quenching by acrylamide is less efficient than at pH 8.0. This results in a smaller Stern–Volmer

Table 1
Stern–Volmer constants of tryptophan fluorescence quenching by acrylamide at different pH values

	K_{SV} (mM^{-1})			
	PC SUVs		PC/PG (1/1) SUVs	
pH	$C_{16}p\text{-}COO^-$	$C_{16}p\text{-}CONH_2$	$C_{16}p\text{-}COO^-$	$C_{16}p\text{-}CONH_2$
8.0	3.6	2.7	3.9	2.9
4.7	3.0	2.6	3.1	3.0
3.6	2.6	2.6	3.0	3.0

Quenching of $C_{16}p\text{-}COO^-$ or $C_{16}p\text{-}CONH_2$, incorporated in SUVs, was determined as described in the legend of Fig. 3.

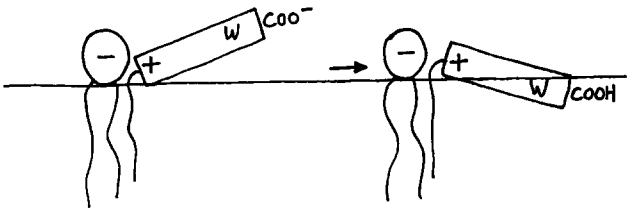

Fig. 4. Schematic representation of anionic lipids-mediated protonation of a negatively charged carboxyl group on a peptide, pre-anchored to a membrane, leading to the movement of this group towards the bilayer interior.

constant (K_{sv}, slope of the Stern-Volmer plot; see also Table 1). A further decrease of the bulk phase pH, to pH 3.6, at which pH the carboxyl group appears to be almost fully protonated (see Fig. 2), results in a further decrease in quenching efficiency. So upon decreasing the pH, the tryptophan residue becomes less accessible to the aqueous quencher, indicating a deeper insertion into the membrane. For $C_{16}p\text{-}CONH_2$ incorporated in DOPC SUVs, the quenching by acrylamide was similar at the different pH values (Table 1). Moreover, the quenching efficiency was comparable to that found for $C_{16}p\text{-}COO^-$ at pH 3.6, indicating that the accessibility of the tryptophan residue for acrylamide of $C_{16}p\text{-}COO^-$ in the fully protonated state is similar to that of $C_{16}p\text{-}CONH_2$. This supports the conclusion, already reached above from the shift in λ_{max}, that the orientation of $C_{16}p\text{-}COO^-$ in the protonated state is similar to that of $C_{16}p\text{-}CONH_2$.

Upon incorporation of 50 mol% PG in the SUVs, the accessibility of $C_{16}p\text{-}COO^-$ to acrylamide also decreased when the bulk phase pH was decreased from 8.0 to 4.7 (Fig. 3B). However, upon a further lowering of the pH, to a value of 3.6, K_{sv} only slightly further decreased, indicating that $C_{16}p\text{-}COO^-$ was already almost fully protonated at the higher bulk phase pH value of 4.7. The accessibility of $C_{16}p\text{-}CONH_2$ was again the same at all three pH values, and comparable to that of $C_{16}p\text{-}COO^-$ at pH 4.7 and pH 3.6, indicating a similar accessibility (Table 1). Comparing the K_{sv} values of the peptides either incorporated in PC SUVs or in the PC/PG SUVs, the values obtained in the PC system are always smaller than those obtained in the PC/PG system. Apparently there is a slight difference in the peptides' accessibility for acrylamide in the two systems, most likely due to the different chemical nature of the interfaces. The results obtained by acrylamide quenching confirm the conclusions drawn from the changes in the λ_{max} and quantum yield of the tryptophan fluorescence.

In conclusion, protonation of a negatively charged carboxyl group on a peptide, pre-anchored to a membrane, leads to the movement of this group towards the bilayer interior, which is schematically depicted in Fig. 4. This results in an orientation which is similar to that of the same peptide without the negatively charged group. Furthermore, it is shown that a negatively charged membrane surface enhances peptide insertion by creating a locally low pH, providing evidence for the hypothesis that anionic lipids can mediate membrane insertion of the anionic part of a bound peptide.

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